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(54) Title: PRODUCTION AND USE OF COMPOSITIONS COMPRISING HIGH CONCENTRATIONS OF VITAMIN B12 ACTIVITY

(57) Abstract

The present invention provides a process for the preparation of a composition comprising natural vitamin B12, wherein said process comprises the steps of: a) obtaining microbial cells containing natural vitamin B12, b) causing opening of the microbial cells such that at least part of the soluble content of the cells comprising vitamin B12 is released in a liquid in which the cells are contained, c) separating the opened cells and the liquid comprising the vitamin B12, d) preparing a mixture of the vitamin B12 and at least a part of the opened cells, wherein the mixture has a vitamin B12 concentration on dry matter in excess of 0.1 % (w/w).

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PRODUCTION AND USE OF COMPOSITIONS COMPRISING HIGH CONCENTRATIONS OF VITAMIN B12 ACTIVITY

Field of the invention

The present invention relates to the production of compositions comprising natural vitamin B12.

Background of the invention

Vitamin B12 is an important vitamin for humans and animals. It is used to treat pernicious anaemia, and peripheral neuritis, and is used as a dietary supplement. Vitamin B12 is also an important animal feed supplement as growth enhancer.

The term vitamin B12 is used to describe compounds of the cobalt corrinoid family, in particular those of the cobalamin group. The most used compound of this group is cyanocobalamin and as such the term vitamin B12 is sometimes used to refer to cyanocobalamin. In this specification the term vitamin B12 should be attributed its broad meaning so as to include all the cobalt corrinoids of the cobalamin group, which include in particular cyanocobalamin, hydroxocobalamin, methylcobalamin and 5'desoxyadenosylcobalamin characterized by a cyano, hydroxyl, methyl or 5'-desoxyadenosyl radical respectively. The methylcobalamin and 5'desoxyadenosylcobalamin compounds are known to be unstable to light in isolated form and are easily transformed to hydroxocobalamin in aqueous solution. For this reason, almost all commercial vitamin B12 preparations consist of the stable cyanocobalamin which as such is not the chemical form in which vitamin B12 can be found in nature. In this specification the term

natural vitamin B12 is defined so as to comprise all chemical forms of vitamin B12 naturally occurring in nature, cyanocobalamin thus being excluded.

Vitamin B12 is produced industrially by microbial fermentation, using almost exclusively *Pseudomonas denitrificans* and *Propionibacterium* species, then converting the natural vitamin B12 into the cyanocobalamin form by chemical processes including cyanidization followed by extraction and purification steps using organic solvents (as reviewed by Spalla *et al*, 1989 "Microbial production of vitamin B12", In: Biotechnology of vitamins, pigments and growth factors, E.J. Vandamme ed., Elsevier, London, New York, pp. 257-284). The chemical conversion step and any subsequent purification steps cause this production process to be expensive, unsafe to the operators and environmentally unfriendly.

Upon ingestion, animals and humans convert cyanocobalamin to one of the natural forms of vitamin B12 such as methylcobalamin or 5'-desoxyadenosylcobalamin which are required to function as a coenzyme for several biochemical conversions (Ellenbogen, L., in: Handbook of vitamins. Nutritional, biochemical and clinical aspects; ed; L.J. Machlin, Marcel Dekker Inc., New York and Basel). In addition efficient growth of animals requires the presence of a sufficient amount of vitamin B12 activity in the animal feed. Vitamin B12 preparations are frequently sold as feed supplements as such or as part of a premix containing additional vitamins and other feed additives.

It is clear therefore that a direct supply of 5'-desoxyadenosylcobalamin or methylcobalamin instead of cyanocobalamin would be of benefit. In view of the instability of these two compounds they are not produced in isolated form but are produced in dry formulation together with the biomass of the organism in which they are produced. Such formulations are well suited for use as animal feed supplement. For the production of methylcobalamin and 5'desoxyadenosylcobalamin, bacteria of the genus *Propionibacterium* are

most preferred, because, unlike *P.denitrificans*, bacteria of this genus have obtained the GRAS (Generally Recognized As Safe) status from the U.S. Food and Drug Administration and are not known to produce endotoxins. *Propionibacterium* species are aerotolerant, nonmotile, nonsporulating, Gram-positive bacteria characterized further by the production of large amounts of propionic acid from carbohydrates, lactic acid and polyhydroxy alcohols. The genus *Propionibacterium* falls into the high "GC" subdivision of the Gram-positive bacteria (T.D. Brock, M.T. Madigan, J.M. Martinko and J. Parker in: The Biology of Microorganisms 7th Edition, Prentice-Hall International Inc., 1994).

Patent application RU 2001953 (Antibiotics Enzymes Research Technical Institute) refers to a spray-drying method for *Propionibacterium shermanii* which has been used to produce 5'-desoxyadenosylcobalamin-containing biomass. Methylcobalamin in biomass from methanogenic bacteria is commercially available from the Gedeon Richter company. However, the vitamin B12 content in these preparations is limited by the level at which the vitamin B12 is produced during fermentation and, as a consequence, the vitamin B12 content in these preparations does not exceed 0.1% (w/w). The concentration of vitamin B12 in a composition is customarily measured as the dry weight of vitamin B12 as a percentage of the dry weight of the composition.

Concentrated (i.e. > 0.1% w/w) products of cyanocobalamin are commercially available. However, feed mills and premix manufacturers cannot use this material because as a consequence of its electrostatic properties cyanocobalamine segregates from its carrier upon processing in feed mills and premix manufacturers when formulated at concentrations > 0.1% w/w. Segregation is a particular problem during sieving, wind sifting or allowing the composition to stand for a prolonged period of time.

The present invention discloses a process by which it becomes possible to produce a composition which has a relatively high concentration (> 0.1% w/w) of natural vitamin B12. These new concentrated products may be used for example in animal feed, human food or as an ingredient in cosmetics. These new concentrated products may be more convenient to use and to transport thus allowing a reduction in costs. They may advantageously be used by feed mills and premix manufacturers as they may show reduced segregation of the vitamin B12 from the carrier during processing. The process can easily be carried out on an industrial scale and is relatively environmentally friendly as there is no need to use organic solvents or cyanidation.

Description of the invention

The present invention provides a process for the preparation of a composition comprising natural vitamin B12 which has a vitamin B12 concentration on dry matter in excess of 0.1% (w/w). Said process comprising the steps of a) obtaining microbial cells containing natural vitamin B12, b) causing opening of the microbial cells such that at least part of the soluble content of the cells comprising vitamin B12 is released into a liquid in which the cells are contained, c) separating the opened cells and the liquid comprising the vitamin B12, and optionally d) preparing a mixture of the vitamin B12 and at least a part of the opened cells.

The microbial cells containing vitamin B12 are preferably obtained in an industrial fermentation process using a microorganism known to produce vitamin B12. These include bacteria belonging to the genera of *Acetobacterium, Aerobacter, Agrobacterium, Alcaligenes, Arthrobacter, Azotobacter, Bacillus, Clostridium, Corynebacterium, Escherichia, Eubacterium, Flavobacterium, Methanobacillus, Methanosarcina, Mycobacterium, Propionibacterium, Proteus, Pseudomonas, Rhizobium, Rhodopseudomonas, Salmonella, Serratia, Streptococcus, Streptomyces* and *Xanthomonas*.

Preferably the bacterium to be used in the process of the invention is safe for consumption by humans and/or animals and does not produce endo- or exotoxins. More preferably the bacterium has obtained the GRAS (Generally Recognized As Safe) status from the U.S. Food and Drug Administration. The most preferred bacterium for use in the process of the invention is of the genus *Propionibacterium*, the preferred species of which are *P.freudenreichii* or *P.shermanii*.

It is well known to persons skilled in the art that the industrial production of vitamin B12, like that of many other microbial metabolites, is carried out using strains resulting from programs designed to improve the desired qualities of any particular strain. These programs consist essentially of the treatment of the production strain with a mutagenic agent and the selection of mutants exhibiting an improved productivity or other advantages. Several techniques have been described for the rational selection of vitamin B12 overproducing mutant microorganisms (Spalla, C., Grein, A., Garofo, L and Ferni, G. Microbial production of vitamin B12 In: Biotechnology of vitamins, pigments and growth factors pp. 257-284 (E.J. Vandamme, ed.) Elsevier, London/NY, 1989). Such mutant strains are used in preferred processes of the invention and are capable of further increasing the vitamin B12 concentrations in the compositions obtained.

In the invention the term "natural vitamin B12" is defined so as to comprise all chemical forms of vitamin B12 naturally occurring in nature, the cyanocobalamin form of vitamin B12 being excluded. In the preferred processes and compositions of the invention the natural vitamin B12 comprises 5'-desoxyadenosylcobalamin and/or methylcobalamin.

In the process of the invention the microbial cells containing vitamin B12 are treated so as to cause lysis or other disruption of the cell membrane. Lysis describes the opening of the microbial cells such that (at least part of) the soluble content of the cells comprising the vitamin B12 is released into a

liquid in which the cells are contained. Preferred treatments for opening the cells are heat treatments including pasteurization or heating in an autoclave; treatment with bacteriolytic enzymes such as lysozyme; mechanical disruption of the cells including grinding or the use of shear forces; treatment with chemicals which cause cell lysis such as detergents or organic solvents; as well as combinations of these treatments. The lysis or other membrane disruption produces a lysate which can be separated into solid and liquid phases.

In a preferred process of the invention at least part of the solid phase (opened cells) is added to the liquid phase containing the natural vitamin B12 so that a composition is obtained which is a mixture comprising biomass, i.e. preferably opened microbial cells, and natural vitamin B12. The vitamin B12 content or concentration of these compositions is expressed as weight-percentage of vitamin B12 based on the dry matter content of the composition.

The process of the invention produces compositions having a vitamin B12 concentration in excess of 0.1% (w/w) on dry matter. In preferred processes the composition has a natural vitamin B12 concentration in excess of 0.2% (w/w), more preferably in excess of 0.4% (w/w), still more preferably in excess of 0.6%, still more preferably in excess of 0.8% (w/w), and most preferably in excess of 1.0% (w/w). It is preferable that vitamin B12 concentrations of the compositions of the invention do not exceed 10% (w/w), more preferably the vitamin B12 concentrations do not exceed 5%.

In the process of the invention the solid phase of the lysate comprising the cell debris resulting from opening the cells is separated from the liquid containing the released vitamin B12. A number of different solid-liquid separation techniques are available to the skilled person for performing this separation, including centrifugation and filtration techniques. A preferred

method for separating the solid cell debris from the vitamin B12 containing liquid is ultrafiltration.

In a preferred process of the invention the opened microbial cells are washed and the washings are combined with the vitamin B12 obtained after separation of the cells and the vitamin B12 containing liquid. Preferably the washing is effected by diafiltration with ion-free water being preferably used for washing the opened cells. The vitamin B12 containing diafiltrate is then combined with the vitamin B12 containing liquid phase.

In a preferred process of the invention further the natural vitamin B12 containing liquid phase is subjected to a drying treatment. Any suitable means of drying can be used such as e.g. spray-drying, fluid-bed drying, freeze drying, or drying in vacuum.

In a preferred process of the invention, the microbial cells containing vitamin B12 are washed prior to undergoing lysis, so as to further increase the vitamin B12 concentration on dry matter by removing medium components. Preferably said washing is performed using diafiltration whereby preferably using ion-free water.

In a further aspect invention provides compositions comprising vitamin B12, which are obtainable in a process of the invention. Preferably the majority of the vitamin B12 activity in these compositions is in the form of natural vitamin B12. Compositions of the invention are characterized in that the vitamin B12 is present in a concentration based on dry matter in excess of 0.1% (w/w). In a preferred composition of the invention the vitamin B12 concentration is in excess of 0.2% (w/w), preferably in excess of 0.4% (w/w), more preferably in excess of 0.6%, still more preferably in excess of 0.8% (w/w), and most preferably in excess of 1.0% (w/w). However, preferably the vitamin B12 concentrations of the compositions of the invention do not exceed 10% (w/w), more preferably the vitamin B12 concentra-

tions do not exceed 5%. The compositions of the invention comprise vitamin B12 and a carrier which preferably comprises biomass e.g. whole cells and/or cell debris. The biomass comprised in these compositions is preferably derived from a microorganism capable of producing vitamin B12, such as the bacteria mentioned above, of which the *Propionibacterium* species are most preferred.

The compositions of the invention are preferably dry compositions, wherein dry is defined as having a water content of less than 15% by weight, more preferably less than 10%, most preferably less than 5%.

In preferred dry compositions of the invention the vitamin B12 activity is distributed substantially homogeneously throughout the powder or granulated powder containing the carrier and the vitamin B12. As result, the vitamin B12 activity in these compositions advantageously does not segregate to a large extent from the other constituents in the composition, especially when exposed to gravitational forces, sieving, wind sifting or electrostatical forces during processing of the compositions, even when used at concentrations in excess of 0.1% (w/w). Processing is herein understood to comprise processing of the compositions of the invention into feed and feed premixes.

In a further aspect of the invention, the composition of the invention is used as or in the production of growth promoting feed supplement for animals. To this end the compositions containing vitamin B12 are added to the other feed components, either directly or in the form of a premix which also contains other vitamins, minerals and/or bioactive ingredients. As shown in the specific example, feeding an animal a diet comprising a composition of the invention promotes its growth.

In a still further aspect of the invention the compositions comprising vitamin B12 are used as or in the production of a human food supplement

and/or are incorporated into cosmetic preparations, such as shampoos or body lotions.

Examples

Experimental

Vitamin B12 assay

Vitamin B12 activity was assayed using the turbidimetric bioassay based on the growth response of *Lactobacillus leichmanii* ATCC 7830 as described in detail in: The United Stated Pharmacopoeia, The National Formulary, 1995, pp. 1719-1721, United Stated Pharmacopoeial Convention, Inc., Rockville MD.

Plasma B12 concentrations

Plasma B12 was measured using a commercially available radioassay kit (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK.). The plasma sample is combined with vitamin B_{12}(^{57}Co) in a solution containing dithiothreitol and cyanide. The mixture is boiled for 30 minutes to inactivate endogenous binding proteins and to convert the various forms of vitamin B_{12} to cyanocobalamin. The mixture is cooled and then combined with immobilised (bound to polymer beads), affinity-purified porcine intrinsic factor. This adjusts and buffers the pH of the reaction mixture to 9.35. The reaction mixture is then incubated for 60 minutes at room temperature. During incubation, the endogenous and labelled vitamins compete for the limited number of binding sites based on their relative concentrations. The reaction mixture is then centrifuged at 1500g for 10 minutes. The labelled and unlabelled vitamins binding to the immobilised binding proteins are concentrated at the bottom of the tube in the form of a pellet and the unbound vitamins remain in the supernatant. The supernatant is aspirated off and the radioactivity associated with the pellet is counted. Standard curves are prepared using vitamin B_{12} standards in a human serum albumin base. The concentration of vitamin B_{12} in the plasma sample is determined from the standard curves. Standard curves were plotted and unknowns determined

using the *AssayZap* universal assay calculator, version 2.32 (Biosoft, Cambridge, UK.).

Plasma samples required to be diluted 10-50 fold to bring them within the most sensitive range of the assay.

Comparative Example 1

A fermentation was performed with *Propionibacterium freudenreichii* using a process known to those skilled in the art (see e.g. Spalla *et al*, 1989 "Microbial production of vitamin B12", In: Biotechnology of vitamins, pigments and growth factors, E.J. Vandamme ed., Elsevier, London, New York, pp. 257-284). A suitable *P. freudenreichii* strain is available from the American Type Culture Collection under accession number ATCC 6207. A broth was obtained at the end of the fermentation which had a 5'-desoxyadenosylcobalamin potency of 10 mg/l and with a dry matter content of 7.5%. Spray-drying of this broth resulted in a product with a vitamin B12 concentration of 0.01%.

In order to obtain a higher vitamin B12 concentration, a solid-liquid separation of the broth was attempted. Vitamin B12 is intracellular in *P. freudenreichii*, for which reason removal (extracellular) medium components and subsequent spray-drying of the biomass should result in a spray-dried product with a higher vitamin B12 concentration.

On lab scale a centrifugation step was performed at a maximal g-force of 5000-6000*g. This g-force is comparable with the centrifugal forces of centrifuges that are used on industrial scale. Aliquots of 1000 ml broth were centrifuged in a Beckmann JM/6E centrifuge during 10 minutes at 5000 rpm. However, no separation of biomass and broth was obtained under these conditions.

Because higher centrifugal forces are less attractive on an industrial scale for economic reasons, we tested whether it is feasible to separate the liquid and solids in the broth using ultrafiltration.

2000 ml of broth, with an initial concentration of 4.5 mg vitamin B12 /l (vitamin B12 on dry matter 0.02 %), was ultrafiltered by means of a 30 kD AMICON spiral wound module (0.09 m^2) with a feed pressure of 1 bar. Under these conditions it was possible to concentrate the sample of vitamin B12 5 times. The average permeateflux was 30 l/m^2h . The vitamin B12 concentration in the concentrate was 20 mg/l, whereas in the permeate less than 0.2 mg/l vitamin B12 was detected. The vitamin B12 concentration in the concentrate, when expressed in terms of dry matter content, was 0.05 %, compared to 0.02% prior to ultrafiltration.

To further increase the titre of vitamin B12 on dry matter, we next tested diafiltration of the biomass.

The ultrafiltration concentrate as obtained in the above described experiment was washed six times with ion-free water, which resulted in a vitamin B12 potency in the concentrate of 20 mg/l. In the diafiltrate no vitamin B12 activity was detected. Subsequent spray-drying of the concentrate resulted in a spray-dried product with a vitamin B12 concentration of 0.06%.

We conclude from the above experiments that it is not possible to obtain a product with a vitamin B12 concentration of more than 0.06% based on dry matter starting from a broth with a vitamin B12 titre of 5 - 10 mg/l.

Example 2

In order to prevent the presence of viable production organisms, i.e. live cells, in the dried product, a pasteurisation step of 65°C for 30 minutes

was performed on a broth which was obtained as described in Example 1. The treated broth was ultrafiltered as described above.

A pink coloured permeate was observed with a vitamin B12 concentration (by dry matter) of 0.2 - 0.4%. The pasteurization is thought to cause lysis of (at least part of) the *P.freudenreichii* cells and release of the intracellular vitamin B12 into the medium. This can allow one to obtain dried products comprising vitamin B12 in concentrations ranging from 0.06% to 0.3% (based on dry matter), by combining the permeate and the pasteurised broth prior to spray-drying.

In more detail 350 ml of the heat-treated broth was ultrafiltered under the conditions described above in Example 1. The concentrate was diafiltered with 2500 ml ion-free water. The clear pink coloured permeate and diafiltrate were combined and mixed with 750 ml pasteurised ultrafiltration concentrate. The mixture was spray- dried in a lab Büchi spray-dryer whereby the inlet temperature was set at 180°C and the outlet was set at a temperature of 104°C. The vitamin B12 concentration of the thus obtained spray-dried product was 0.13%

Example 3

1200 l of broth with a vitamin B12 concentration of 8 mg/l was obtained in a pilot plant fermentation of *P.freudenreichii* and was ultrafiltrated in a DDS ultra filtration module M37 using 20 kD membranes and an initial waterflux of 38 l/m^2h. The temperature during ultrafiltration was kept ambient. The circulation flow was about 15 m^3/h. The feed pressure was set at about 7 bar. The broth was concentrated 11.5 times (average flux of 23 l/m^2h). The concentrate was washed with 500 l of ion-free water at ambient temperature till a conductivity of about 3 mS/cm was reached.

The resulting concentrate was treated at 90°C for 6 hours. Part of the heat-treated concentrate was diafiltered, resulting in about 300 l of vitamin B12 containing permeate. The retentate was stored at 4°C.

The permeate was concentrated on a pilot scale in a glass evaporator at 40 °C under vacuum, resulting in about 6 l of concentrate.

By combining and mixing an appropriate amount of the retentate with the concentrated permeate, we composed a mixture which was spray-dried in a NIRO spray-dryer. The air temperature at the inlet was set at 160°C and the outlet temperature was regulated at about 90 - 95 °C. In 5 hours operation time about 45 l of the mixture was spray-dried (about 9 kg/hour). This resulted in 1307 g of spray-dried material with a vitamin B12 activity of 1100 mg/kg, i.e. 0.11%.

Example 4

In an experiment similar to the one as described in Example 3, a fermentation was performed using the improved *P.freudenreichii* strain CBS 929.97. The resulting vitamin B12 concentration in the fermentation broth was 40 mg/l. The ultrafiltration and diafiltration were essentialy performed as described in Example 3.

Using ultrafiltration, 100 l of the 40 mg/l broth was concentrated to about 40 l. Using diafiltration, the concentrate was washed with 200 l of ion-free water. The resulting washed concentrate had a vitamin B12 activity of 96.5 mg/kg and 6.72% of dry matter (spray drying of this washed concentrate would have given a vitamin B12 activity of 0.14%).

The washed concentrate was subsequently lysed at 90°C for 5 minutes, followed by a separation of cell debris and liquid by ultrafiltration. The potential vitamin B12 concentration of the permeate reached of 0.87% at dry matter (claculated from a vitamin B12 activity of 52.1 mg/kg and a dry matter content of 0.6%). After preconcentration of the permeate in vacuum at 65°C, different amounts of permeate and concentrate were combined and

dried. The vitamin B12 potencies of the obtained dried products were as given below:

concentrate : permeate	vitamin B12 activity by dry matter
1 : 1	0.5%
3 : 7	0.65%
2 : 8	0.72%
8 : 2	0.29%

P.freudenreichii CBS 929.97 was deposited 10 July, 1997 at the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands.

Example 5

Application of natural vitamin B12 in animal feed.

A trial was performed with broilers to compare the efficacy of natural vitamin B12 with cyanocobalamin. One day old male broilers were randomly assigned to cages. Eight animals were kept per cage. The cages were situated in an artificial heated, ventilated and illuminated broiler house. The animals were vaccinated against New Castle disease at ages one and fourteen days. The animals received the experimental diets *ad libitum* from day 1, the first 10 days in the form of crumbles, later in the form of pellets. Water was available freely. The experiment lasted 28 days. At day 28 the animals were weighed, feed consumption determined and blood samples were taken. The blood samples were taken from 4 randomly selected broilers per cage. Blood was collected in heparinised tubes. The tubes were centrifuged and the plasma was frozen (-18 °C) until vitamin B12 analysis was carried out. This analysis was performed as described above under Experimental (Plasma B12 concentrations).

Three treatments were comprised in this experiment:

I. Basal diet without addition of vitamin B12
II. Basal diet with addition of cyanocobalamin (30 ppb active substance)
III. Basal diet with addition of natural vitamin B12 (30 ppb active substance)

Each treatment was replicated 5 times.

The composition of the basal diet is presented in table 1, the results in table 2.

Table 1. Composition of the basal diet (in %).

Maize	55
Tapioca	1
Soybeanmeal	30
Soybeans, heat treated	5
Feather meal	1
Soya oil	2
Animal fat	3
Vitamins*, minerals, amino acids	3

Calculated contents:

Metabolic Energy	13.3 MJ/kg
Crude protein	22.0 %
Lysine (total)	1.27 %
Methionine + Cysteine (total)	0.95 %

*: Without vitamin B12.

Table 2. Effect of cyanocobalamin and natural vitamin B12 on average growth, feed intake and feed conversion ratio between 1 and 28 days of age and on blood plasma vitamin B12 contents at day 28.

	Growth (g)	Feed intake (g)	Feed conversion ratio	Bloodplasma Vit. B12 (ng/l)
Basal diet	1139	1738	1.53	14
+ Cyanocobalamin	1264	1782	1.41	32
+ Natural vitamin B12	1261	1771	1.40	41

Example 6

Segregation of vitamin B12 activity from a carrier

Segregation of the active compound from the carrier may be caused by particle shape, density and bulk density, flowability, adhesive and electrostatic properties, moisture content, and hygroscopicity. These segregation processes may occur during manufacturing of premixes and compound feed and during subsequent transport and storage.

This segregation is particularly critical in the case of premixes which comprise microcomponents such as vitamins and trace elements, macrocomponents such as minerals and carriers.

The demixing properties of existing commercial cyanocobalamin preparations (obtained from Rhône Poulenc under the brandname Microvit® B12) has been compared with natural vitamin B12 preparation prepared as described in example 4.

Limestone is used as a carrier for the existing commercial cyanocobalamine preparations.

The test substances were run out of a storage vessel via a vibrating channel to form a pile as shown in Figure 1.

Three samples were taken from the center and mixed. The procedure was repeated with samples from the base of the pile. The samples were analyzed for vitamin B12 activity as described above and compared withe the original homogeneous mix. Deviations in samples from the original homogeneous mix are calculated as follows:

Deviation at base (%) = {(concentration as base - concentration original mix)/ concentration original mix} * 100

Deviation at center (%) = {(concentration at center - concentration original mix)/ concentration original mix} * 100

Comparison of the deviations at the pile base and pile center of compositions of cyanocobalamin and natural vitamin B12 of the invention are shown in Table 3.

Table 3.

Test substance	Separation test (%) Pile base	Separation test (%) Pile center
Cyanocobalamin 0.1%	< +10	< -10
Cyanocobalamin 1%	+56	-78
Natural vitamin B12 (0.9%)	+8	-12

PCT-2800

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 14, line 18/19 of the description [1]

A. IDENTIFICATION OF DEPOSIT [2]

Further deposits are identified on an additional sheet [ ] [3]

Name of depositary institution [4]

Centraal Bureau voor Schimmelcultures (CBS)

Address of depositary institution (including postal code and country) [4]

Oosterstraat 1
P.O.Box 273
3740 AG BAARN (The Netherlands)

Date of deposit [5]	Accession Number [6]
10 July 1997	CBS 929.97

B. ADDITIONAL INDICATIONS [1] (leave blank if not applicable). This information is continued on a separate attached sheet [ ]

We inform you that the availability of the microorganism identified above, referred to Rule 13bis PCT, shall be effected only by issue of a sample to an expert nominated by the requester until the publication of the mention of grant of the national patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn.

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [1] (If the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS [1] (leave blank if not applicable)

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E. [ ] This sheet was received with the international application when filed (to be checked by the receiving Office)

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was 12 AUG 1997 (12.08.97) Y. Marinus-v.d. Nouweland

(Authorized Officer)

Form PCT/RO/134 (January 1981)

Claims

1. A process for preparing natural vitamin B12 the process comprising:

(a) culturing microbial cells under conditions such that they produce, intracellularly, natural vitamin B12; and

(b) lysing or otherwise disrupting the outer membrane of the microbial cells that have produced natural vitamin B12 so as to release the vitamin B12.

2. a process according to claim 1 wherein the lysis produces a lysate and the solid and liquid phases of the lysate are separated, the liquid phase containing the natural vitamin B12.

3. A process according to claim 2 which further comprises washing the solid phase of the lysate and combining the washings with the liquid phase of the lysate.

4. A process according to claim 3 wherein the washing of the solid phase of the lysate is effected by diafiltration.

5. A process according to any one of claims 2 to 4 which further comprises adding at least part of the solid phase of the lysate to the liquid phase containing the natural vitamin B12.

6. A process according to any one of the preceding claims which further comprises washing the microbial cells prior to lysis.

7. a process according to claim 6 wherein the washing of the microbial cells prior to lysis is effected by diafiltration.

8. A process according to any one of claims 2 to 7 which further comprises drying the liquid phase containing the natural vitamin B12.

9. A process according to any one of the preceding claims wherein the microbial cells are cells from one or more of the bacterial genera *Acetobacterium, Acetobacter, Agrobacterium, Alcaligenes, Arthrobacter, Azotobacter, Bacillus, Clostridium, Corynebacterium, Escherichia, Eubacterium, Flavobacterium, Methanobacillus, Methanosarcina, Mycobacterium, Propionibacterium, Proteus, Pseudomonas, Rhizobium,*

Rhodopseudomonas, Salmonella, Serratia, Streptococcus, Streptomyces and *Xanthomonas*.

10. a process according to claim 9 wherein the microbial cells are cells of the bacterial genus *Propionibacterium*.

11. a process according to any one of the preceding claims wherein the lysis is caused by heat treatment, pasteurisation, treatment with bacteriolytic enzymes, mechanical disruption or chemical treatment, either singularly or in combination with each other.

12. A process according to any one of claims 2 to 11 wherein separation of the solid and liquid phases of the lysate is effected by ultrafiltration or microfiltration.

13. A process according to any one of the preceding claims wherein the natural vitamin B12 is 5'-desoxyadenosylcobalamin or methylcobalamin.

14. A composition comprising natural vitamin B12 in an amount greater than 0.1% weight based on the dry matter content of the composition.

15. A composition according to claim 13 which is obtainable by the process defined in any one of claims 1 to 13.

16. A composition according to claim 14 or 15 which is a dry composition wherein the natural vitamin B12 is distributed substantially homogenously throughout the composition and wherein the vitamin B12 does not segregate from the other components when exposed to sieving, wind, sifting, gravitational forces or electrostatic forces.

17. A composition according to any one of claims 14 to 16 which comprises biomass.

18. A composition according to claim 16 wherein the biomass is derived from bacteria of the genera *Acetobacterium, Acetobacter, Agrobacterium, Alcaligenes, Arthrobacter, Azotobacter, Bacillus, clostridium, corynebacterium, Escherichia, Eubacterium, Flavobacterium, Methanobacillus, Methanosarcina, Mycobacterium, Propionibacterium, Proteus, Pseudomonas, Rhizobium, Rhodopseudomonas, Salmonella, Serratia, Streptococcus,*

Streptomyces and *Xanthomonas* either singularly or in combinations of two or more.

19. A composition according to claim 18 wherein the genus is *Propionibacterium*.

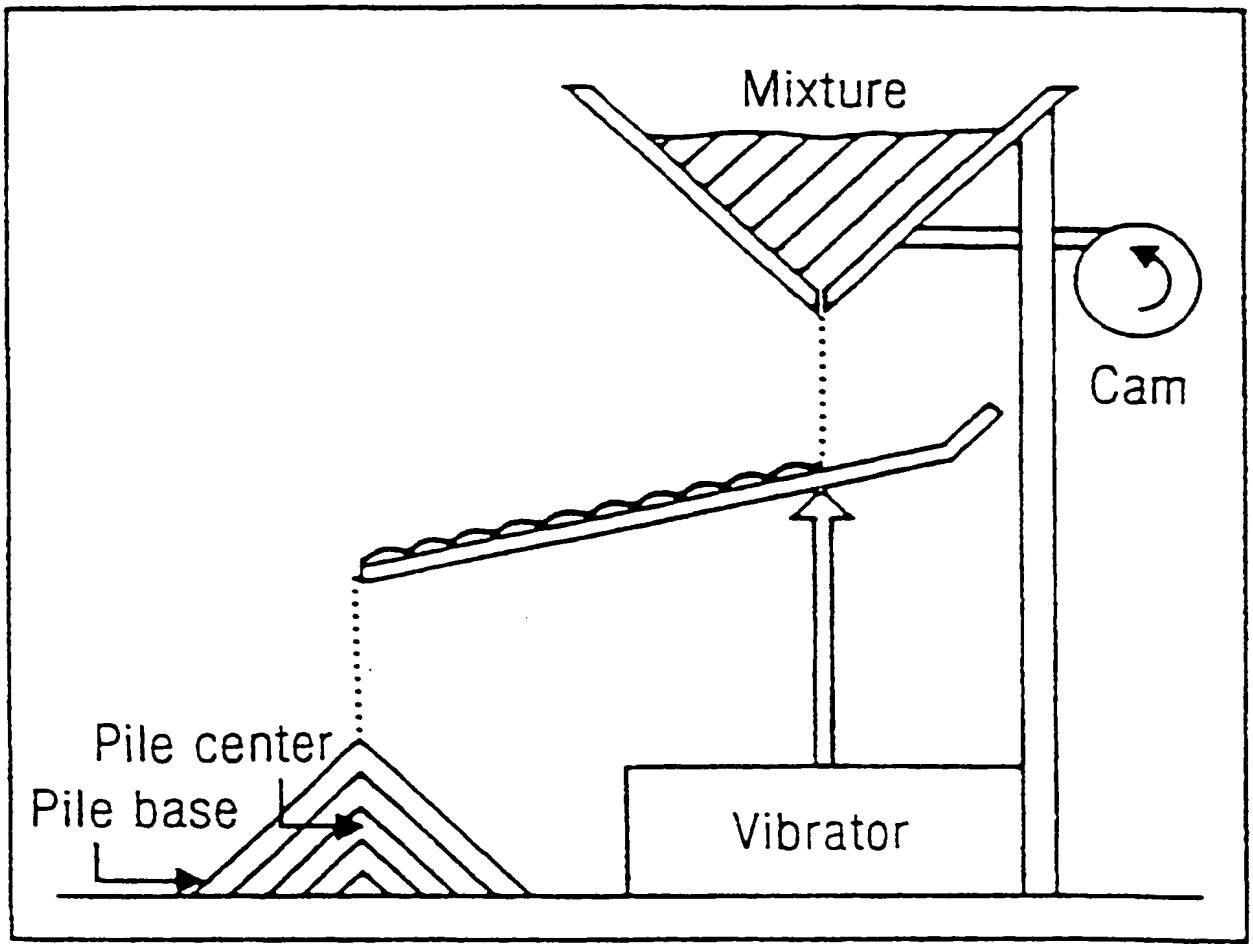
20. A composition according to any one of claims 14 to 19 wherein the natural vitamin B12 is 5'-desoxyadenosylcobalamin or methylcobalamin.

21. Use of a composition according to any one of claims 14 to 20 in the production of a food supplement.

22. Use of a composition according to any one of claims 14 to 20 in the production of a cosmetic preparation.

Figure 1

Separation test



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(57) Abstract

The present invention provides a process for the preparation of a composition comprising natural vitamin B12, wherein said proces comprises the steps of: a) obtaining microbial cells containing natural vitamin B12, b) causing opening of the microbial cells such that a least part of the soluble content of the cells comprising vitamin B12 is released in a liquid in which the cells are contained, c) separatin the opened cells and the liquid comprising the vitamin B12, d) preparing a mixture of the vitamin B12 and at least a part of the opene cells, wherein the mixture has a vitamin B12 concentration on dry matter in excess of 0.1 % (w/w).

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INTERNATIONAL SEARCH REPORT

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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHEMICAL ABSTRACTS, vol. 83, no. 5, 4 August 1975 Columbus, Ohio, US; abstract no. 41434b, FUKUI SABURO ET AL.: "Production of vitamin B12 by methanol assimilating bacteria" XP002024719 see abstract	1-22
X	& ASAHI GARASU KOGYO GIJUTSU SHOREIKAI KENKYU HOKOKU, vol. 24, 1974, JP, pages 231-241, --- -/--	1-22

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

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1

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHEMICAL ABSTRACTS, vol. 84, no. 3, 19 January 1976 Columbus, Ohio, US; abstract no. 14412g, KVASINOV, E.I. ET AL.: "Synthesis of B vitamins by bacteria of the genus Arthrobacter." XP002024720 see abstract	1-22
X	& MIKROBIOL. ZH., vol. 37, no. 5, 1975, KIEV, pages 573-579, ---	1-22
X	ATSUO TANAKA ET AL.: "Production of vitamin B12 by methanol-assimilating bacteria." J. FERMENT. TECHNOL., vol. 52, no. 12, 1974, pages 921-924, XP000614245 see the whole document ---	1-22
X	SCHLIWA M. ET AL.: "Stabilzation of the cytoplasmic ground substance in detergent-opened cells and a structural and biochemical analysis of its composition." PROC. NATL. ACAD. SCI., vol. 78, no. 7, 1981, USA, pages 4329-4333, XP000614241 see the whole document ---	1-22
X	HARUKI MORIKAWA AND TADASHI KAMIKUBO: "Utilisation of hydrocarbons by microorganisms." J. FERMENT. TECHNOL., vol. 47, no. 8, 1969, pages 470-477, XP000614246 see the whole document ---	1-22
X	KATSUHIKO FUJII ET AL. : "Studies on the formation of vitamins and their functions in hydrocarbon fermentation." HAKKO KOGAKU ZASSHI, vol. 44, no. 4, 1966, pages 185-191, XP000614244 see the whole document -----	1-22